The role of Aspirin and its additive effect along with Vitamin C on the development of gastric tumours induced by carcinogen (Nitrosopiperidine) in rats

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A Thesis submitted to The Tamil Nadu Dr. M. G. R. Medical University

In partial fulfillment of the degree M. S. (Branch 1) General Surgery

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Certificate

This is to certify that **"The role of Aspirin and its additive effect along with Vitamin C on the development of gastric tumours induced by carcinogen (Nitrosopiperidine) in rats"**, which is being submitted as thesis requirement for M.S. Degree Branch I – General Surgery examination of The Tamil Nadu Dr. M. G. R. Medical University, is a bonafide work of the candidate – **Dr. Shalom Sylvester Andugala**

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This is to certify that the topic entitled **"The role of Aspirin and its additive effect along with Vitamin C on the development of gastric tumours induced by carcinogen (Nitrosopiperidine) in rats"** is a bonafide work done by **Dr. Shalom Sylvester Andugla**, post graduate in General Surgery of Christian Medical College, Vellore. This work has been carried under my guidance and supervision in partial fulfillment of the regulation of The Tamil Nadu Dr. M. G. R. Medical University - Branch I (General Surgery) examination to be held in March 2009.

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Aims and Objectives

1. **To create an animal model for carcinogen induced gastric cancer in rats**

2. **To Study the effect of aspirin and vitamin C in modifying the progression of gastric cancer induced by carcinogen in the animal model**

Introduction

Gastric cancer is one of the leading causes of death amongst cancer patients. It is surpassed only by lung cancer as the most common of the malignancies. Delay in the diagnosis of gastric cancer is one the main causes for its lethal nature. The pathogenesis of gastric cancer is a complex process involving different stages of development in malignancy ranging from chronic active gastritis to dysplasia and metaplasia and eventually, malignancy. A number of causative factors have been implicated in the transformation of normal gastric mucosa into malignant cells. Environmental factors include infection by H. pylori, presence of nitrites in the food which act as carcinogens (nitrosamines) and lack of fresh fruit and vegetables. Host factors include chronic gastritis and partial gastrectomy status which favors reflux of bile and alkaline intestinal secretions. Patients with blood group A have been found to be more susceptible to gastric cancer. A positive family history, hereditary non-polyposis colon cancer syndrome and familial gastric carcinoma syndrome (E-cadherin mutation) suggest that genetic factors seem to have a role in the predisposition to gastric cancer. Among the environmental factors, an important one seems to be exposure to carcinogen, perhaps in the diet. There is a world wide geographic distribution of gastric cancer

with higher incidence in countries like Japan and some parts of South America and lower incidences in Western Europe and United States. Studies done on migrant population, those who have moved in to areas of low incidence from areas of high incidence, have shown that environmental, genetic and cultural factors seem to influence predisposition to gastric cancer. (1)

This study aims at producing a gastric cancer model in rats using a carcinogen to induce malignancy. As carcinogens, mainly nitrosamines, in the diet have been implicated in causing gastric cancer; this study aims at using a carcinogen, a nitrosamine, to induce gastric cancer in rats. With such a model in mind, the study also aims to look at the influence of Aspirin and Vitamin C in modifying the progression of gastric cancer induced by the carcinogen.

The results of this study, if found to be significant, could be used for further study in a human model. As gastric cancer propagates through various stages of development, could Aspirin be used to alter the progression of disease if picked up early by gastroscopy is the question under study. The implications of this study being that if a patient has been diagnosed to have any early features of malignancy which may include

chronic atrophic gastritis or dysplasia , does long term intake of aspirin prevent progression of disease?

This study aims to determine if this would be possible on an animal model.

Review of Literature

Carcinogen:

The most important of the environmental factors responsible for causing gastric cancer seems to be exposure to carcinogens, more specifically nitrosamines. Numerous nitrosamine compounds have been studied and animal experiments have been conducted to study the carcinogenic properties by Lijinsky and many others (2). Approximately 300 different Nitrosamines have carcinogenic potential. Different animal species show carcinogenic response to these chemicals. Exposure to sufficient quantities of these compounds can also induce cancer in humans. Exposure to these compounds can occur through diet, occupational exposure, use of tobacco products, cosmetics, pharmaceutical products and agricultural chemicals (5).

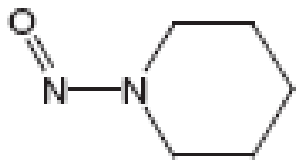
Mechanism of action:

Extensive studies of these compounds have shown that they, by themselves are not carcinogenic. However, after enzymatic oxidative metabolism, they are converted into a precursor of the final carcinogen.

There is considerable evidence to show that damage to cellular DNA and proteins by electrophiles generated from these metabolites may be responsible for their characteristic biological effects. Certain DNA base lesions, particularly Guanine O^6 alkylation are apparently more critical in giving rise to cell transformation than others, e.g. N_7 alkylation. It has been demonstrated that, of the possible sites of enzymatic oxidation, hydroxylation at the positions α to the N-nitroso group ultimately produces the carcinogen. The resulting α-hydroxymethylnitrosamines have been suggested to lose formaldehyde readily to generate the primary alkylnitrosamine which tautomerizes to the alkanediazohydroxide. The latter being a powerful electrophile alkylates sensitive cellular nucleophiles, including DNA base sites. The α-acetoxymethylalkylnitrosamines are especially pertinent since they have been shown to be potent carcinogens. (6).

In this study, the carcinogen chosen was Nitrosopiperidine, a member of the nitrosamine group.

N-Nitrosopiperidine



Properties:

N-Nitrosopiperidine is a yellow oil. It is soluble in water, organic solvents, lipids, and hydrochloric acid. It decomposes when exposed to light, and is especially sensitive to ultraviolet light. When heated to decomposition, it emits toxic fumes of nitrogen oxides. It is oxidized by strong oxidants to the corresponding nitramine and it can be reduced to the corresponding hydrazine and/or amine. It is relatively resistant to hydrolysis, but can be reduced by hydrogen bromide in acetic acid

Carcinogenicity:

N-Nitrosopiperidine is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity in experimental animals.

When administered in the diet, *N*-nitrosopiperidine induced squamous cell carcinomas of the forestomach, papillomas of the esophagus, hepatocellular adenomas and carcinomas, and liver hemangioendotheliomas in male mice. When administered in drinking water, *N*-nitrosopiperidine induced lung adenomas in mice of both sexes and esophageal carcinomas and hepatocellular carcinomas in rats. When administered orally, *N*-nitrosopiperidine induced hepatocellular carcinomas in monkeys. When administered by subcutaneous injection, the compound induced squamous cell carcinomas and other tumours of the nasal cavity, esophageal squamous cell carcinomas and papillomas in rats. Tumours of the nasal cavity, trachea, lung, tongue, palate, esophagus, forestomach, and liver were seen in hamsters. When administered by intraperitoneal injection, *N*-nitrosopiperidine increased the incidence of adenomas of the lung in mice. When administered by intravenous injection, the compound induced carcinomas of the esophagus and pharynx in rats. When administered to pregnant hamsters, a low incidence of tumours of the upper respiratory tract was observed for the offspring and a high incidence of respiratory tract tumours was observed for the mothers (IARC 1978, 1987).

So far there are no adequate human studies on the relationship between exposure to *N*-nitrosopiperidine and cancer.

Use :

N-Nitrosopiperidine is used as a research chemical; no other uses have been identified.

N-METHYL-N'-NITRO-N-NITROSOGUANIDINE (MNNG)

Amongst the carcinogens used in experiments to induce stomach cancer, MNNG is the most important one, used exclusively to induce gastric cancer. MNNG induced DNA strand breaks in various organs of rats treated in vivo. It did not cause dominant lethal mutations in mice, but it gave positive results for mutation in the mouse spot test; it induced chromosomal aberrations and micronuclei in bone-marrow cells of mice and sister chromatid exchanges in bone-marrow cells of mice and Chinese hamsters treated in vivo. It induced chromosomal aberrations, sister chromatid exchanges, DNA strand breaks and unscheduled DNA synthesis in human and rodent cells in vitro and induced mutation in cultured rodent cells. It gave positive results in several assays for cell transformation. MNNG induced somatic and sex-linked recessive lethal mutations in Drosophila. It caused chromosomal aberrations, sister

chromatid exchanges and mutation in plants and recombination and mutation in fungi. It was mutagenic to and caused DNA damage in bacteria, and gave positive results in host-mediated assays using bacteria or yeast as indicators and mice as hosts (7).

MNNG has been tested for carcinogenicity in mice, rats, hamsters, rabbits and dogs, producing tumours at many sites. It has a predominantly local carcinogenic effect and is carcinogenic in single-dose experiments. Following its oral administration, papillomas and squamous-cell carcinomas of the oesophagus and forestomach, adenocarcinomas of the stomach, small intestine and large bowel, and sarcomas of the gastrointestinal tract were reported. These findings have been extended in more recent studies after oral administration to rats, hamsters and dogs. After subcutaneous injection in mice, it produced lung and liver tumours and haemangioendotheliomas; after intrarectal, intrauterine and intravaginal application in rats and guinea-pigs, it produced local tumours. (7).

P.J. Hu and his colleagues conducted an experiment on 86 male wistar rats using MNNG to induce stomach cancer and study the chemopreventive effect of Celecoxib. This compound, when administered

orally with drinking water induced stomach cancer in 75% of the rats given MNNG only (8).

This study was initially designed using MNNG as the carcinogen. However, due to the nonavailability of this drug, another carcinogen, Nitrosopiperidine was chosen.

The Rat:

Rats are the most commonly used animal for conducting experiments, next only to the mouse. Animal studies on rats include those involving Nutrition, transplantation, immunology, genetics, pharmacology and cancer research, to name a few.

Rat strains descendent from Wistar:

(a) Direct

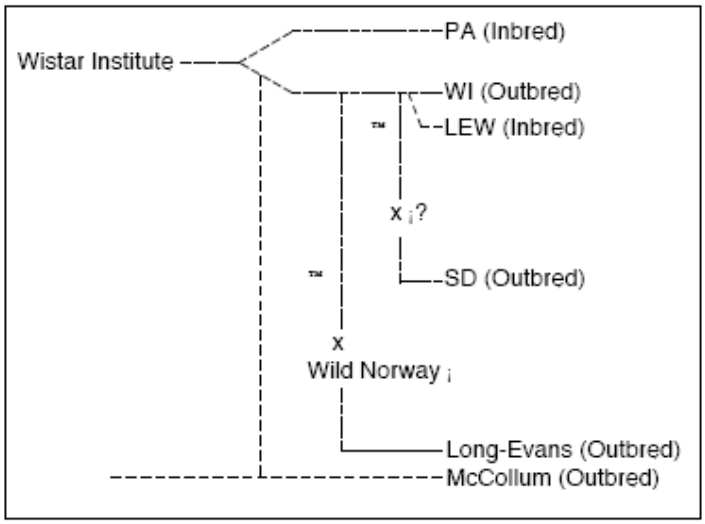
AS	K	MNE	SHR	WE
B	Kyn	MNRA	W	WF
BRUFO	LEW	MR	WA	WKy
BuF	LOU/c	OKA	WAB	WM
GH	LOU/m	RA	WAG	WN

(b) From crosses

BD(11-x)	IS	McCollum
BDE	LE	Sprague-Dawley
BS	LGE	
GHA	MAXX	

From Lindsey (1979).

Genealogy of out bred rat strains:



General characteristics of Rats:

Rats have a well developed diurnal rhythm. They are active during the dark and rest during the light hours. Feeding occurs in the night and digestion during the early daylight hours. During this period, caged rats can easily be handled. The mean life span of male out bred rats is around 1000 days and females 1300 days but inbred generally have shorter life spans.

Wistar rats are an out bred strain of albino rats. They belong to the species Rattus norvegicus. This strain was originally developed in the Wistar Institute. Its purpose was mainly for biological and medical research, and is notably the first rat strain to be developed to serve as a model organism. The large size of the rat is apt for conducting procedures which would be difficult to perform in mice. Most laboratory rat strains are descended from a colony of rats established at the Wistar Institute in 1906 by Henry Donaldson, Milton. J. Greenman and Helen Dean King. The Wistar rat is currently one of the most popular rat strains used to laboratory research. It is characterized by its wide head, long ears, and having a tail length that is always less than its body length. The Sprague Dawley rat and Long-Evans rat strains were developed from Wistar rats.

Resistance to tumour induction:

Hiroko and colleagues conducted experiments in rats to study their genetic susceptibility to gastric carcinoma after treatment with carcinogen, MNNG. They found that resistance to induction of gastric adenocarcinoma by MNNG was a dominant characteristic (9). There are a few resistant strains of rats that resist the induction of tumours. Can this trait be isolated and then studied in humans? The answer to this question can only be obtained by continual research at molecular level to study the genes responsible for these traits.

Basic biological data

Life span		2–3.5 yr
Weights		
Birth		5–6 g
Weaning		30–55 g
Puberty		150–200 g
12 weeks	Male	200–400 g
	Female	150–270 g
Adult	Male	300–800 g
	Female	250–400 g
Hair coat		9 days
Eyes open		12–14 days
Ear canal open		12–14 days
Descent of testes		15–50 days
Oestrous cycle		4–6 days
Gestation		21–22 days
Litter size		6–14
Maximum milk yield		12–14 days
Weaning age		20–21 days
Rectal temperature		38–39°C
Heart rate		320–480 bpm
Blood pressure	Diastolic	60–90 mm Hg
	Systolic	75–120 mm Hg
Respiratory rate		85–110 breaths/min
Tidal volume		1.6 ml
Body surface area (cm^2)		9.1 $kg^{0.66}$
Daily food consumption		5 g/100 g bwt
Daily water consumption		8–11 ml/100 g bwt
Urine output per day		5.5 ml/100 g bwt
Urine osmolality		1660 mOsm/kg of H_2O
Urine pH		7.5–8.5
Urine SG		1.04–1.07
Blood volume		5.6–7.1 ml/100 g bwt
Plasma volume		3.08–3.67 ml/100 g bwt
Red blood cell		7–10 x $10^6/\mu l$
Haemoglobin		11–19 gm/dl
Packed cell volume		40.5–54%
Clotting time		2–5 min
Prothrombin time		8–4 sec
PTT		21.1 ± 3.7 sec
Fibrinogen		190 (150–230) mg/dl
Leukocyte count		
Total		9(6–18) x $10^3/\mu l$
Neutrophils		14–20%
Lymphocytes		69–86%
Monocytes		1–6%
Eosinophils		1–4%
Basophils		Rare
Platelets		500–1,000 x $10^3/\mu l$

From Baker *et al*. (1979), Weihe (1987).

Environmental requirements for the comfort and well being of laboratory rats and mice (From Clough, 1992):

Factor	Range	Relevance to comfort and well-being of rats and mice
Temperature	23 ± 5°C	Not likely to be stressful or have major adverse effects.
Relative humidity	55 ± 15%	As above if achieved in cages 55 ± 10% (already adopted by some authorities) allows greater margin for removal of water from cages.
Ventilation rate	8–20 ac/h	15 ac/h in fully stocked rooms probably satisfactory provided it is associated with efficient air distribution system. New systems may allow fewer air changes in room.
Light intensity	60–400 lux	350–400 lux satisfactory for staff working. Care needed to avoid retinal damage to albinos in upper cages.
Photoperiod	12L:12D	Not likely to be stressful or have major adverse effects.
Wavelength		Lack of information of wave-length effects but no evidence that daylight-type fluorescent or tungsten lights have adverse effects.
Sound	~50dB(A) & NRC45 to <85dB	Current recommendations all related to human ear function and are irrelevant to animal hearing. This factor is the one most likely to give rise to discomfort and lack of well-being in these species.

Non–Steroidal Anti-inflammatory Drugs – Their Role in Chemo-prevention

Patients with Rheumatoid arthritis were found to have lower incidences of gastrointestinal malignancies (3, 4). Many studies have shown this to be true. This was thought to be due to the ingestion of Non Steroidal Anti-inflammatory Drugs. This theory was proved by both observational and controlled epidemiological studies in patients with colo-rectal cancer.

In a population based case-control study K. Akre and colleagues, in 5 Swedish counties, interviewed 567 incident cases of gastric cancer and 1165 controls were interviewed. The cases were uniformly classified to subsite (cardia or non-cardia) and histological type and information collected on other known risk factors for gastric cancer. Users of Aspirin had a moderately reduced risk of gastric cancer compared to never users (odd ratio adjusted for age, gender and socio-economic status was 0.7 (95 % CI = 0.6 – 1.0)). Gastric cancer risk fell with increasing frequency of aspirin use ($p = 0.02$). The risk reduction was apparent for both cardia and non-cardia tumours but not so certain for the diffuse type. No other association was observed between gastric cancer and non-aspirin NSAIDs or other studied pain relievers. These findings led to the hypothesis that use of aspirin reduces the risk of gastric cancer (10).

Results from an American population based case-control study showed odds ratios of 0.55 for gastric adenocarcinomas and 0.88 (not statistically significant) for gastric cardia adenocarcinoma among regular users of aspirin, compared to never users (Farrow et al, 1998). With the same exposure categories, a hospital–based case-control study from Moscow reported an odds ratio of 0.49 and 1.14 (not statistically significant) for non-cardia and cardia cancer, respectively (Zaridze et al, 1999).

The relationship of Aspirin use and esophageal cancer was examined using data from the National Health and Nutrition Examination Survey (NHANES 1) and the National Epidemiologic Follow-up Studies (NEFS). Persons were classified as nonusers, occasional users or regular users of aspirin based on their response to two questions at the base-line examination: whether they had taken aspirin in the last 30 days and whether they had used pain medications regularly during the prior 6 months. Occasional use was associated with a 90 % decreased risk (95% CI, 0.01-0.76) of developing esophageal cancer; none of the regular users developed the disease. Adjusting for cigarette smoking (ever .vs. never) and alcohol intake (at least monthly .vs. not) did not explain the findings (11).

C57BL/6 mice were treated with a carcinogen N-methyl Nitrosourea (MNU) and/or H. pylori. Nimesulide (NSAID) was mixed with feed pellets and administered for the duration of the experiment. Gastric tumours developed in 68.8 % of mice that were given both MNU and H. pylori, where as less than 10% developed gastric cancer when given either MNU or H. pylori alone. In mice treated with both MNU and H. pylori, nimesulide administration substantially decreased H. pylori associated gastric tumorigenesis, where as substantial inductions of apoptosis were observed. (12).

This property of Aspirin and non-aspirin NSAIDs was thought to be due to its inhibitory action on Cyclo-oxygenase II enzyme.
Kirsi Saukkonen and his colleagues studied the expression of COX-2 expression in human gastric dysplasias and adenocarcinomas. Performance of several COX-2 antibodies was evaluated, after which COX-2 protein expression was studied in 67 gastric cancer specimens and in 8 definite dysplasias by using immunohistochemistry. COX-2 positivity was detected in 58% of the intestinal type (well differentiated) tumours and 6 % of diffuse –type (poorly differentiated) tumours. There was a higher expression of COX-2 mRNA, protein and enzymatic activity in well-differentiated gastric cancer cell lines when compared with poorly differentiated cell lines. COX-2 immuno-reactivity was localised to the

carcinoma cells, the stroma of the tumours was negative. Strong COX-2 positivity was consistently detected in stromal cells at sites of erosions and ulcerations. Moreover 44 % definite dysplasias of the stomach that showed no evidence of invasion were positive for COX-2(13).

Having proved that there is over expression of COX-2 by the cancer cells, it was thought that the mechanism of action of aspirin and non-aspirin NSAIDs was by inhibition of this enzyme. After the discovery of over expression of COX-2, selective COX-2 inhibitors were being used in experiments to study their chemo-preventive role.

P. J. Hu et al divided 86 male Wistar rats into 6 different treatment groups.

Group A – Water alone (n=5)

Group B – N-methyl-N'-nitro-N-nitrosoguanidine (MNNG 100 microgram/ml) (n=16)

Group C – Indomethacin (3mg/Kg/day) (n=16)

Group D – Celecoxib (5mg/Kg/day) (n=17)

Group E - Celecoxib (10mg/Kg/day) (n=16)

Group F - Celecoxib 20mg/Kg/day) (n=16)

Group B-F animals were treated with 10% sodium chloride (in the initial 6 weeks) and MNNG in drinking water to induce adenocarcinoma in the

stomach. All animals received treatment for 40 weeks, and were sacrificed after death or at 48 weeks. Gastric neoplasm was evaluated by histology. The incidences of gastric cancer were 0%in group A, 75% in group B, 68.8% in group C, 70.6% in group D, 18.8% in group E and 31.3 % in group F. Compared with MNNG controls, treatment with celecoxib 10mg/kg/day also showed lower tumour multiplicity and lower mean tumour volume. The tumours had significantly higher COX-2 expression than their adjacent normal tissues ($p < 0.02$). There was no significant difference in COX-2 levels among tumours in the different treatment groups. Lower tumour prostaglandin E2 level was found in the indomethacin treated group, suggesting that the chemo preventive effect of celecoxib may be mediated by a COX-2 independent pathway (14).

Having proven that COX-2 expression is much higher in these tumours, and that treatment with selective COX-2 drugs does decrease the tumour load, it may sound logical to give COX-2 inhibitors to all patients diagnosed to have early pre-cursor lesions of gastric cancer or those who are at a higher risk of developing gastric cancer.

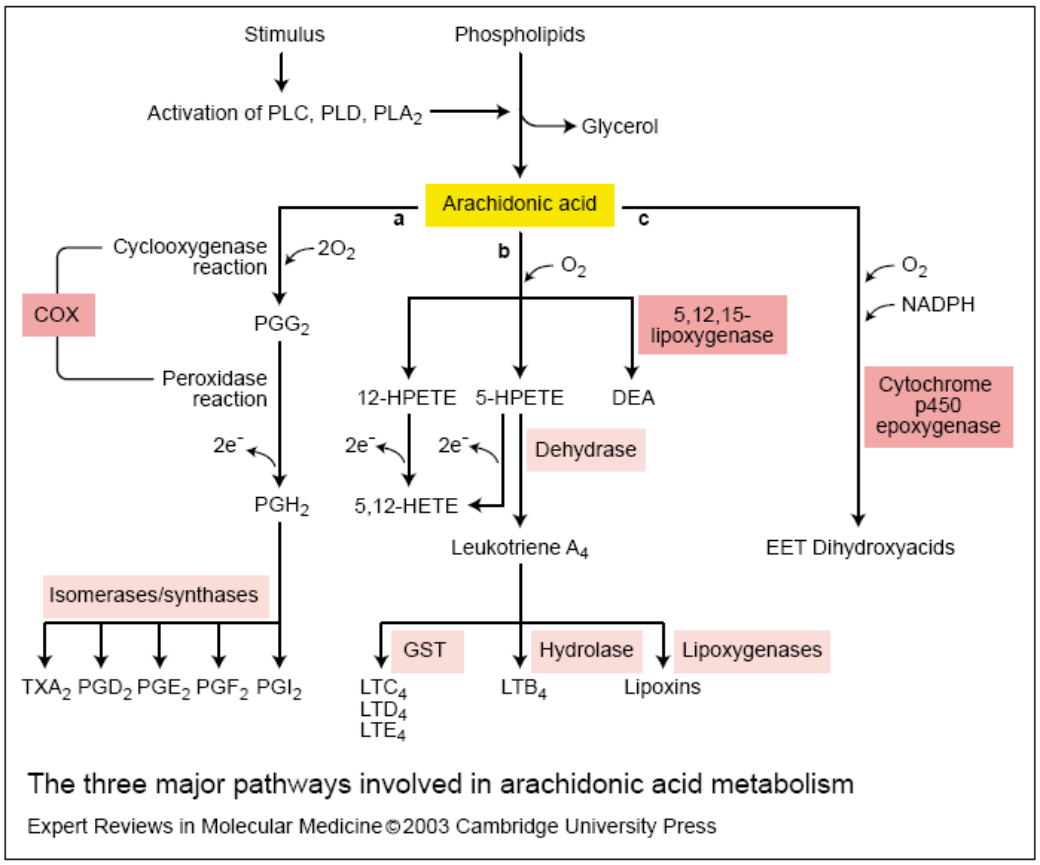
VIGOR (Vioxx Gastrointestinal Outcomes Research Study) study, which was initially designed to assess whether rofecoxib is associated with a lower incidence of clinically important upper gastrointestinal events than naproxen in patients with rheumatoid arthritis, showed the relative risk of

developing a confirmed adjudicated thrombotic cardiovascular event (myocardial infarction, unstable angina, cardiac thrombus, resuscitated cardiac arrest, sudden or unexplained death, ischaemic stroke and transient ishcaemic attacks) with rofecoxib treatment in comparison to that with naproxen was 2.38 (p= 0.002) (15). Rofecoxib was withdrawn from the market. In an APC cancer trial, the drug company Pfizer demonstrated an increased cardiovascular risk over placebo for celecoxib and thus the drug was withdrawn. The US FDA issued a Public Health Advisory, which stated that the long term use of NSAIDs and selective COX-2 inhibitors might increase the risk of severe cardiovascular events.

With this background, the role of aspirin, a drug very commonly used especially in patients with history of cardiovascular events was chosen to study its chemo protective properties.

Aspirin and its mechanism of action –

Aspirin exerts its effects predominantly through its antiprostaglandin activity. Cyclooxygenase (COX) is the key enzyme in the synthesis of prostaglandins from arachidonic acid, which is inhibited by aspirin.



The three major pathways involved in arachidonic acid metabolism

Two isoenzymes have been identified for cyclooxygenase – Cyclooxygenase 1 (COX 1) and Cyclooxygenase 2 (COX 2). Aspirin covalently modifies both COX 1 and COX 2 thus causing an irreversible inhibition of Cyclooxygenase activity. Duration of action is related to the

turnover rate of Cyclo-oxygenases in different target tissues. Aspirin acetylates serine 530 of COX 1 and prevents binding of arachidonic acid to the active site of the enzyme. It acetylates serine 516 of COX 2 leading to the formation of 15(R)- hydroxyl eicosatetraenoic acid which is converted to 15 – epilipoxin A4 and this potentiates the anti-inflammatory action of Aspirin (Goodman and Gillman's Pharmacological Basis of Therapeutics – 10^{th} ed.).

Cyclooxygenase 1 (COX-1) is constitutively expressed and considered to be a housekeeping gene, while Cyclooxygenase 2 (COX-2) is not usually detectable in normal tissues, but can be readily induced in processes like inflammation, reproduction and carcinogenesis. The mechanisms by which COX-2 is thought to be involved in the carcinogenesis include (16)

a) Resisting apoptosis

b) Increasing cell proliferation

c) Stimulating angiogenesis

d) Modulating the invasive properties of cancer cells

COX-2 is inducible by oncogenes ras and scr, interleukin-1, hypoxia, benzo[a]pyrene, ultraviolet light, epidermal growth factor, transforming growth factor beta, and tumour necrosis factor alpha. Dexamethasone, antioxidants, and tumour-suppressor protein p53 suppress COX-2 expression. COX-2 synthesizes prostaglandin E2 (PGE2) which stimulates bcl-2 and inhibits apoptosis, and induces interleukin-6 (IL-6) which enhances haptoglobin synthesis. PGE2 is associated with tumour metastases, IL-6 with cancer cell invasion, and haptoglobin with implantation and angiogenesis (17).

A clear positive correlation between COX-2 expression and inhibition of apoptosis has been established, associated with increased PGE2 levels resulting in modulation of pro- and anti-apoptotic factors (e.g., bcl-2, MAKs/ras, caspase-3, Par-4). In terms of angiogenesis and invasiveness, COX-2 activity was found to increase the expression of growth factors (e.g., VDEG, PDGF, bFGF) and matrix metalloproteinases (MMPs) (18).

Prostaglandins – Their role in cancer development

Maxwell et al confirmed that the blood from the veins draining the tumor especially from the gastrointestinal tract is found to have high prostaglandin E2. There was no statistically significant difference in the prostaglandin biosynthetic capacity of epithelial cells derived from tumour tissue compared to epithelial cells derived from uninvolved tissue. However significant difference in biosynthetic capacity was observed between tissue fixed mononuclear cells derived form tumours compared to uninvolved tissue. There was no difference in prostaglandin E2 synthesis observed between peripheral mononuclear cells derived from colon cancer patients or from normal individuals. The aggregates of these observations suggest that the host cells rather than the tumour cells may be the major source of prostaglandins that contribute to tumourogenesis (19).

Possible mechanism of malignant transformation and growth linked to prostaglandin (PG) synthesis –

1) Mutagenesis – Melonaldehyde is a breakdown product of prostaglandin H2, which acts as a mutagenic as well as carcinogenic agent. Aspirin, by inhibiting Cyclooxygenase, reduces the synthesis of prostaglandins, so melonaldehyde production is reduced as it a byproduct of PG breakdown (19).

2) Prostaglandin synthesis dependant carcinogen activation – PG synthase dependant xenobiotic metabolism has been demonstrated in several intact cellular systems. Aromatic amines, heterocyclic amines and dihydrodiol derivatives of polycyclic hydrocarbons are activated to mutagenic derivatives by PG synthase. Aspirin, by inhibiting PG synthetase, decreases these mutagenic derivatives (19).

3) Cell growth – Experimental animal studies have shown that mammary epithelial cell lines are stimulated by Thromboxane A2 and inhibited by thromboxane A2 inhibitors. Thromboxane A2 is a breakdown product of prostaglandins, so aspirin, by blocking the

synthesis of prostaglandins, reduces the production of thromboxane A2 (19).

4) Tumour promotion – PG are found to have a role in tumour promotion in the epidermis of mice by phobol esters. Phorbol esters are present in the epidermal cells. Administration of tumour promoting phorbol esters to mouse skin in cultured murin keratinocytes triggers the release of arachidonic acid, which will lead on to PG synthesis. Aspirin, by inhibiting the PG synthesis from arachidonic acid, reduces tumour production. (19).

5) Immune response – Another mechanism by which cyclooxygenase inhibitors may decrease tumourogenesis is by modulation of immune response. Colony stimulating factors from macrophages activate monocytes to produce PG E2, which inhibits the blastogenesis of T-cells and cytotoxic activity of natural killer cells. This appears to be part of a generalized response of the immune system to the presence of foreign antigen (19).

6) Anti-platelet action – Aspirin prevents platelet aggregation to tumour cells thereby preventing the platelet tumour cell aggregate to get attached to the capillary endothelium. By this, the lodging of

tumour cells gets prevented. They also provide an immunological shield to the tumour cells. The efficacy of low dose aspirin as an antiplatelet suggests that inhibition of platelet function should be considered as a mechanism for inhibition for cancer mortality. Metastatic disease is frequently the cause of death from cancer and inhibition of metastasis certainly has a significantly beneficial effect on the mortality of colon cancers. (20).

Thun et all studied that platelet is the only cell type sensitive to inhibition by low to moderate dose of aspirin, but slow to cyclooxygenase activity. The importance of PG especially thromboxane A2 and its role in the regulation of platelet function is the basis for a reason for interrupting the metastatic cascade. Although there are some reports indicating the lack of an anti-metastatic effect on aspirin, it may be related to difference in dosage, route of administration, preparation of tumour cells on metastasis used (20).

Recent studies have shown that Aspirin induces apoptosis (21, 22) thus exerting its anti-tumourigenic action.

Vitamin C, antioxidants and free radicals:

Free radicals are chemicals with a single unpaired electron in their outer orbit. They are extremely unstable and readily react with organic or inorganic chemicals. They attack and degrade nucleic acids as well as a variety of membrane molecules. They also initiate autocatalytic reactions. Molecules that interact with these free radicals are in turn converted to free radicals thus propagating chain of damage.

Free radicals can be generated in cells by

a) Absorption of radiant energy (x-rays, UV rays)
b) Reduction-oxidation reactions that occur during normal physiologic process.
c) Enzymatic metabolism of exogenous chemicals

Three reactions are particularly relevant to cell injury mediated by free radicals:

1. Lipid peroxidation of membranes : Double bonds in membrane polyunsaturated lipids are vulnerable to attack by oxygen-derived free radicals

2. Lesions in DNA : Reactions with thymine in nuclear and mitochondrial DNA produce single strand breaks which has been implicated in both cell death and malignant transformation
3. Cross-linking of proteins: These radicals promote sulfhydryl-mediated protein cross-linking, resulting in enhanced rates of degradation or loss of enzymatic activity. They may also cause poly peptide fragmentation.

Free radical formation is also a part of the normal physiology including microbial defence and respiration. Therefore the cells have developed mechanisms to minimize the injury produced by these generated free radicals. Free radicals are inherently unstable and generally decay spontaneously. Superoxide dismutase significantly increases the rate of decay of these free radicals. Another enzymes, such as glutathione peroxidise, also protect against injury. Exogenous and endogenous antioxidants (Vitamin E) may either block free radical formation or scavenge them once they are formed.
(Basic Pathology. Kumar, Cotran, Robbins)

Generation of reactive oxygen species is another important causative factor for production of gastric cancer. The gastro intestinal tract is particularly susceptible to reactive oxygen species attack which leads to

carcinogenesis. Antioxidants play an important role in preventing tumour induction.

Skrzydlewska and his associates studied the activity of superoxide dismutase, catalase, glutathione peroxidise, reductase, the level of glutathione, vitamin C, malondialdehyde and cancer procoagulant in tumours and nomal mucosa from 18 patients with esophageal cancer, 18 patients with gastric tumour and 62 patients with colorectal cancer. An increased activity of these enzymes was noted. In gastric cancer patients, the activity of glutathione peroxidase and reductase was decreased. There seemed to be an imbalance in the antioxidant potential which led to increase in reactive oxygen species action. (13).

Skrzydlewska also found a statistically significant increase in the level of lipid peroxidation products in patients with colorectal cancer. Also the levels of Vitamin C and E were also reduced thereby proving that colorectal carcinogenesis was associated with serious oxidative stress and that gradual advancement of oxidative-antioxidative disorders was followed by progression of colorectal cancer. (24).

From various animal studies, it has been clearly shown that an imbalance in the regulation of regeneration of free oxygen radicals leads to the formation of malignancy.

Gastric juice contents were analyzed in one study to assess the levels of ascorbic acid, total bile acids, nitrite, nitrate and total nitroso compounds (NOCs) in 56 patients with non-operated stomachs undergoing endoscopy for dyspepsia. It was found that patients with chronic atrophic gastritis had lower gastric juice ascorbic acid concentrations, higher pH than normal subjects which were significant. Patients with reflux gastritis had higher total bile acid concentrations and lower gastric ascorbic acid concentrations than those with chronic gastritis and no intestinal metaplasia. There was however no significant differences in plasma vitamin C or gastric nitrite, nitrate or total NOC concentrations in relation to gastric histology. (25).

The role of ascorbic acid (Vitamin C) in cancer production has been unclear. Though there are studies that show low levels of Vitamin C in patients with gastric cancer, there are others that show that vitamin C intake enhances gastric cancer production. However, because of its antioxidant properties, its role as a protective substance has also been considered in this study.

Materials and Methods

This experiment was conducted on the Wistar breed of rats. The animals were obtained from the National Centre for Biological Sciences, Bangalore. Total number of rats required to conduct this experiment was 60. This sample size was obtained using the data from the study conducted by P.J. Hu et al on rats where gastric cancer was induced using a carcinogen.

Approval of the college research board and the animal ethics committee was obtained before the commencement of the study. The rats were divided in to 3 groups, each group having 20 rats. The rats were caged together in fours. The experiment was conducted in the Animal Laboratory under controlled environmental conditions. The room temperature was maintained at 24 to 26 degrees. Humidity was maintained at 50 to 60 %. The light cycle period was 14 hours of day and 10 hours of night. The rats were given regular rat feeds along with supply of fresh water. The feed was a standard rat feed and was available ad libitum. Bedding changes were made twice weekly. Polypropylene cages were used to house the animals. A veterinary doctor was available to monitor the health of the rats.

Group A	N = 20
Group B	N = 18
Group C	N = 20

(2 rats had died within 12 weeks of initiating the experiment)

The rats were initially quarantined. After dividing them in to their respective groups, they were fed with regular food pellets and water for one week duration for acclimatization after which the experiment was initiated. Carcinogen, aspirin and vitamin C were all given with drinking water.

Drug delivery:

The first group received Carcinogen only along with drinking water. The second group received carcinogen and aspirin, both mixed with drinking water. Dispensable aspirin tablets were used. The third group was given aspirin, vitamin C and the carcinogen. The carcinogen was initially dissolved in the appropriate solvent and then made into the required concentration and packaged in vials. Each vial contained 38 mg of the carcinogen for every 5 ml of solution. The food and water were changed

everyday. The respective drugs were mixed in water and distributed equally for each rat and cage – 40 ml of the solution for each rat. Any left over solution was discarded and fresh solution was prepared for the next day. If the assigned drug-water solution was consumed completely before 24 hours, fresh drinking water was supplied without the drug(s).

A period of 12 weeks was allotted for assessment of drug over dosage or drug toxicity. Rats dying within 12 weeks of initiating the experiment were not considered in the study and this was thought to be due to drug toxicity.

The initial plan was to carry out the study for seven months. How ever as there were no tumours detected in the specimens of the rats that had died before the end the experiment or were sacrificed at the end of 7 months, the duration of the experiment was extended to one year with a higher concentration of the carcinogen, given along with drinking water and prepared by the same method.

At the end of 7 months, 11 rats were initially sacrificed but as there was no evidence of malignancy, both in gross appearance and on histological examination, the experiment was continued for another 5 months, a total of one year.

Autopsy and Histopathological Examination:

The rats were first subjected to anaesthesia. High dose of chloroform in a closed container was used. After inhalation of the anaesthetic gas at such high doses, death occurred within 3-4 minutes. After this the autopsy was performed. The abdominal viscera were examined for any evidence of tumours. One of the rats developed a liver mass. The stomach along with the esophagus (distal 2/3) was removed. Gross examination of the tissues was done for tumours. Half of the tissue that was removed was fixed in formalin for histopathology examination. The remaining tissue was stored for further immunohistochemisty studies.

The specimen was first fixed in 10% formalin for 24 hours. Representative samples were taken by the pathologist and these were placed in cassettes. These cassettes were placed in an automated Histokine machine where there were subjected to different concentrations of alcohol (70%, 80%, 90%), Touludine or Acetone, 2 baths of paraffin and finally Xylene. During this process, the tissue gets embedded in paraffin and the paraffin impregnates in to the tissue. The paraffin blocks were cooled on ice. The paraffin blocks were cut to a thickness of 5 microns using a microtome. The sections were placed in a hot water bath

and transferred on to a glass slide. Glycerine was used to transfer the sections on to the glass slide. These were then subjected to incubation at 37 degrees for 45 minutes following which H&E staining was done.

Results

Both gross and histological examination failed to reveal the presence of any malignancy. Pre-cancerous conditions such as dysplasia also were not found in any of the tissues. The carcinogen used failed to induce any malignant changes in the tissues.

Group A – Gross	Group A – Microscopic	Group B – Gross	Group B – Microscopic	Group C – Gross	Group C - Microscopic
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
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-	-			-	-
-	-			-	-

Discussion

There were two deaths during the initial 12 weeks. The exact cause of death could not be identified. Autopsy of these rats did not reveal any evidence of gross tumour but histopathological evaluation for any evidence of microscopic tumour was not possible as the rats had died overnight and had already begun to decompose. These rats were not included in the study.

During the course of the experiment, there was a period of sudden death of these rats occurring over a very short period. The autopsy performed on these rats did not however reveal any features of malignancy, both on gross examination and on histological examination. As rats being used for other experiments also began to die, a common factor such as temperature regulation was thought to be the cause for these sudden deaths. After the temperatures were strictly regulated, these deaths stopped occurring. However there were other cases of deaths that occurred even after this. As the animals were caged in groups of four, there were occasional conflicts amongst the members of the same cage resulting in death of one of the animals as confirmed by bite marks. This was not a common phenomenon and hence they remained in groups.

Another interesting phenomenon that occurred was loss of hair and ulceration of the foot pad with loss of toes. This was not restricted to any one group. A veterinary doctor was consulted for this condition and this was thought to be due to a skin infection. Despite necessary medications, this condition did not seem to improve with medications. Also, as the duration of the experiment was prolonged, the age factor was also considered to be responsible for the hair loss.

This study eventually did not produce any positive results. The carcinogen used failed to induce any features of malignancy in the tissues including microscopic changes such as dysplasia. Hence the effect of aspirin and vitamin C could not be evaluated. The following have been considered to be responsible for the above results.

1. The carcinogen used in the experiment was not a very potent carcinogen in terms of its carcinogenic effect on gastric tissues. The initially planned carcinogen was N-Methyl-N ‘-Nitro-N-Nitrosoguanidine (MNNG) which is a very potent carcinogen and used very extensively in animal experiments to produce a gastric cancer in animal models, especially rats. As this compound was not available, an alternative compound was used in the study. The failure of this

compound to induce cancer may have also been due to the fact that during shipping of this product, its potency may have been lost. Also the potency may have been lost during storage of this compound. Another reason may have been due to inadequate dosage used. Perhaps a higher dose of the carcinogen should have been used. This was thought of after 7 months, and a much higher dose was used for 5 months. Despite this modification in the dosage no features of malignancy were found.

2. Resistant strains: Resistant laboratory animals (rat strains) have been bred which have shown resistance to development of cancer induced by carcinogen. Experiments conducted by Hiroko and colleagues in rats showed that resistance to induction of gastric adenocarcinoma by carcinogen (MNNG) was a dominant characteristic in certain strains of laboratory bred rats (5).

Presence of resistant strains leads us in a new direction of research – the possible mechanisms of resistance. What are the underlying molecular mechanisms that promote resistance to cancer production and can this information be used to produce resistance to cancer in humans.

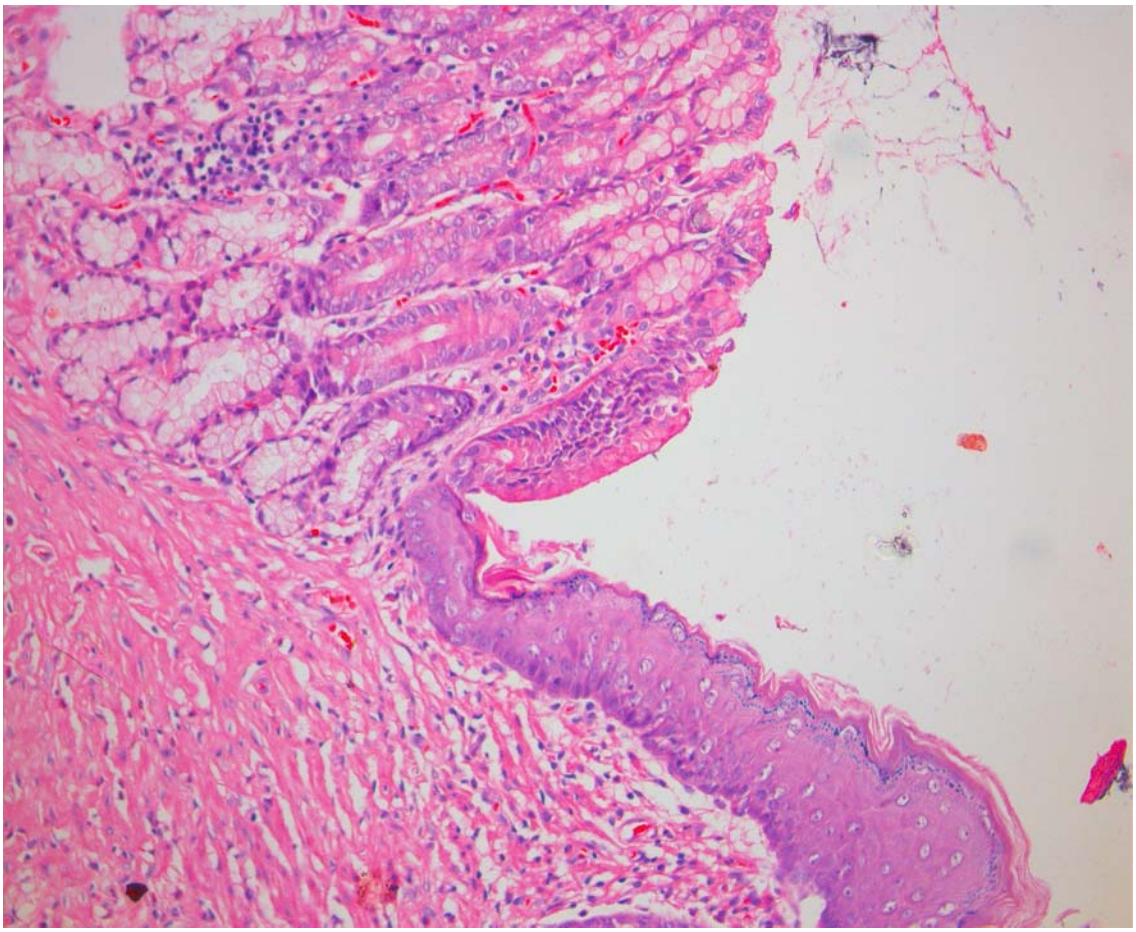
(Pic 1 – At the end of the experiment)

(Pic 2 – Loss of hair over the head)

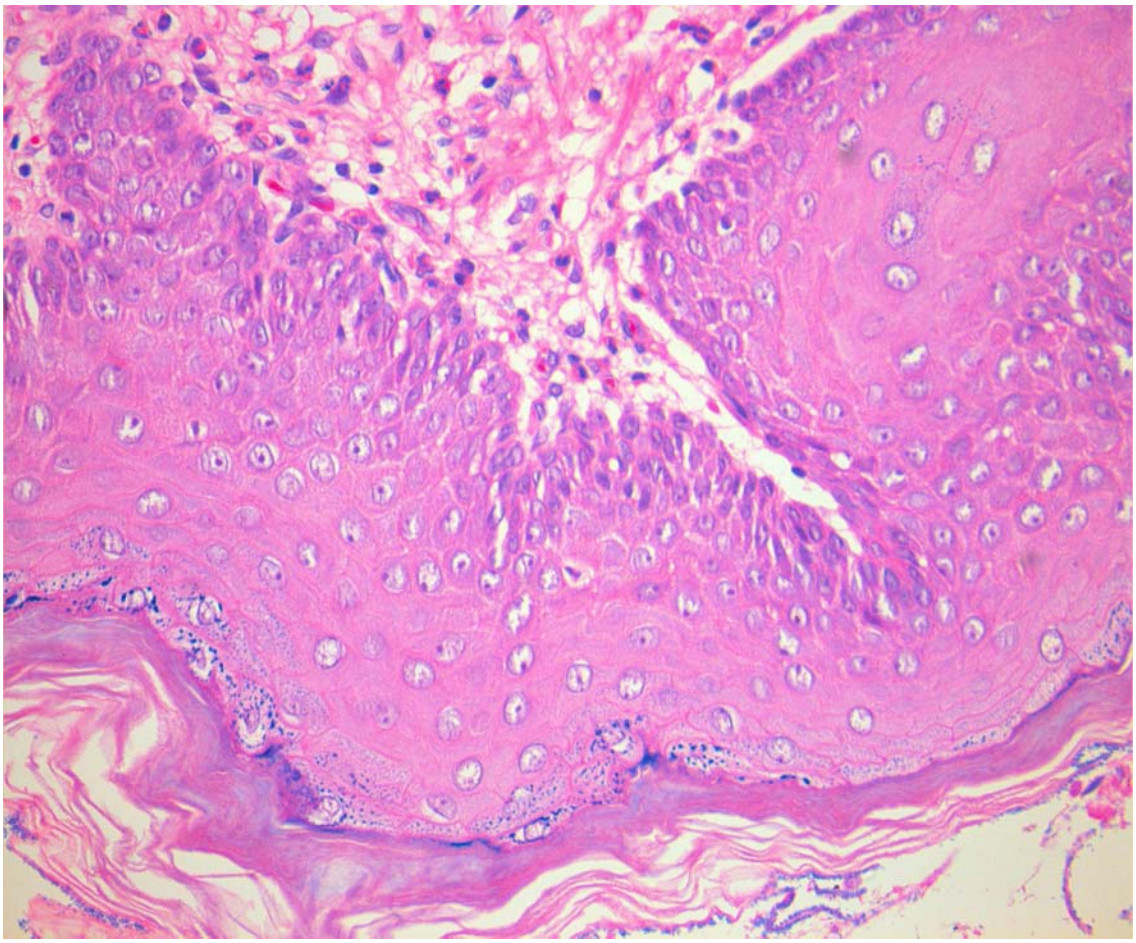
(Pic 3 - Foot ulceration)

(Pic 4 - Hair loss over the body)

Medium Power view of gastro-esophageal junction showing keratinized esophageal epithelium



High power view of esophageal epithelium showing mild dysplasia and no evidence of malignancy



Conclusions

In conclusion, the negative results of this study can be attributed to two factors –

a) The carcinogen used in the experiment was not a very potent carcinogen in terms of its carcinogenic effect on gastric tissues.
b) It is possible that resistant laboratory animals (rat strains) have been bred which have shown resistance to the development of cancer induced by carcinogen.

Mankind continues to struggle for survival in his war against cancer. Though extensive research has been done in the field of cancer science, both in its etiopathogenesis and also in its treatment, search for better and appropriate modalities of treatment continues. Some of them such as gastric cancer are silent killers and detected quite late. Preventive measures may be instituted in those that are at high risk or in those early pre-cancerous lesions have been detected. Finding preventive measures will go a long way in saving lives than finding a cure.

Prevention is better than cure!

Future directions

Further studies could be conducted in the future to produce an animal model for gastric cancer. Though such models have been successfully created using MNNG compound, experiments using other nitrosamine compounds could be conducted to produce these animal models and this model be used to study the characteristics of gastric cancer at a molecular level including genetic studies, such as its induction, progression and metastasis. Experiments utilizing surgical procedures that produce reflux of duodenal contents in to the stomach could also be conducted to study the effect of reflux on gastric cancer induction. Such information could be used further to find a cure for the disease or a preventive measure.

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